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13. ABSTRACT (Maximum 200) The data presented in this annual report (for award DAMD17-96-1-6022) support our hypothesis for a mechanism of retinoic acid -induced growth arrest of human breast cancer cells. Specifically we believe that retinoic acid induced growth arrest of human breast cancer cells requires protein kinase Ca expression. The constitutive expression of PKCa in hormone independent, MDA-MB-231 cells induced retinoic acid sensitivity to inhibit the uncontrolled proliferation and to alter the proto-oncogene expression. Retinoic acid treatment in PKCa expressing MDA-MB-231 cells reduced 40% of cell proliferation and inhibited serum induction of c-fos expression. Concomitantly, c-jun expression was enhanced. These findings, along with the observation that retinoic acid treatment enhanced PKCa expression, conventional PKC activity and translocation of PKCa to cell membrane and nucleus, raised the possibility that PKCa induced retinoic acid sensitivity in MDA-MB-231 cells is caused by disturbance of mitogenic signaling or transcriptional regulation of target genes. By turning on/off the transcription of PKCa gene, we further demonstrated the PKCa regulation of c-fos and c-jun expression. In total, we demonstrate that the lack of anti-proliferation effect of retinoic acid in MDA-MB-231 cells is caused by the failure of RA induced PKCa expression and subsequent absence of PKCa altered gene expression.				
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INTRODUCTION

Retinoic acid (RA) inhibits the proliferation of many, but not all, human breast carcinoma cells lines (Liu et al., 1996; Wilcken et al., 1996). The anti-proliferative action of RA appears to be limited to hormone-dependent breast carcinoma cells, in which RA inhibits estrogen stimulation of proliferation (Liu et al., 1996; Wilcken et al., 1996). Estrogen's mitogenic effects are believed to result from increased expression of autocrine peptide growth factors and surface receptor tyrosine kinases (Daly et al., 1994; Halter et al., 1992). RA inhibits signaling between receptor tyrosine kinases and the nucleus (Leid et al., 1993; Talmage and Listerud, 1994). The components of these signaling pathways that serve as targets for RA have yet to be identified. At least two signaling pathways stimulated by receptor tyrosine kinases utilize Protein Kinase C (PKC) family members. These include phospholipase C γ derived second messenger activation of the conventional PKCs, PKC α , β and γ , and novel PKCs, PKC δ and ϵ (Blobe et al, 1994), and activation of the atypical PKC ζ by the phosphatidylinositol 3-kinase product, PtdIns 3,4,5-P $_3$.

Retinoic acid (RA) induced growth arrest is frequently associated with changes in PKC isozyme expression. In our preliminary data (Cho et al., 1997), RA treatment of hormone dependent T-47D breast cancer cells, but not hormone independent MDA-MB-231 breast cancer cells resulted in increased PKC α and decreased PKC ζ expression. Because of the relationships between hormonal dependent mammary cell proliferation, RA growth regulation and protein kinase C, **we have hypothesized that retinoic acid inhibits the uncontrolled proliferation of mammary carcinoma cells, by increasing the overall activity of PKC α and/or decreasing the overall activity of PKC ζ .**

Summary of previous findings (Year 1)

Task 1. Proliferation of the hormone dependent, T-47D human breast carcinoma cells was inhibited by retinoids. All-trans retinoic acid (RA), and the RAR α -selective synthetic retinoid, Am580 were equally effective at arresting growth of T-47D cells. The similar dose response for growth arrest seen between RA and Am580 argue for a primary role of RAR α regulating mammary cell proliferation. In contrast, hormone independent breast carcinoma cells, MDA-MB-231 was insensitive to RA for growth arrest.

Untreated T-47D cells expressed the novel PKC isozymes, PKC δ and PKC ϵ , and the atypical PKC ζ , but not conventional PKCs (PKC α , β or γ). When T-47D cells were treated with RA, PKC α expression was induced and concomitantly PKC ζ expression decreased. In contrast to T-47D cells, RA treatment of MDA-MB-231 cells had no effect on PKC α or PKC ζ expression.

The prompt expression of PKC α following RA treatment coincided with the cessation of proliferation in T-47D cells. When proliferation of T-47D cells was assayed in the presence of Am580, Gö6976, a selective inhibitor of conventional PKCs (cPKCs), or both, addition of Gö6976 prevented the Am580 induced reduction in proliferation. Therefore retinoid arrested proliferation of T-47D cells required the activity of conventional PKCs, presumably the retinoid-induced PKC α .

Task 2. During year 1 of this project, we were establishing T-47D and MDA-MB-231 cells stably expressing PKC α , PKC ζ , antisense PKC α and antisense PKC ζ . After stable transfection is established, we planned to complete Task 2 in each cell line

including control T-47D and MDA-MB-231 cells. Stable transfection of Sense/Antisense PKC α in MDA-MB231 cells and data from these cell lines are reported below.

Task 3. After stable transfection of PKC α , PKC ζ , antisense PKC α and antisense PKC ζ , in T-47D and MDA-MB-231 cells, we plan to characterize retinoic acid effect on cell cycle progression in each cell line.

Task 4. In order to determine the functional role of PKC α in mediating the proliferative response of T-47D cells to retinoids, we isolated T-47D cells constitutively expressing PKC α following infection with a recombinant retrovirus (pSVX α). SVX α infected T-47D cells (α T-47D) expressed PKC α in the absence of retinoic acid. No additional alterations in PKC β , γ , δ , ϵ and ζ were seen in α T-47D cells. Constitutive expression of PKC α slowed T-47D proliferation more effectively than 10^{-8} M RA treatment. Treatment of α T-47D cells with the selective inhibitor of cPKCs, Gö6976 increased proliferation to near control levels.

By using two human breast carcinoma cells lines; the hormone (estrogen)-dependent T-47D cell line originated from a ductal carcinoma and the hormone (estrogen)-independent MDA-MB-231 cell line, originated from an adenocarcinoma, the data presented in first annual report support our hypothesis for a mechanism of RA-induced growth inhibition of T-47D cells. Induction of PKC α expression and concomitant repression of PKC ζ expression following RA treatment are consistent with RA inducing growth arrest of T-47D cells. In contrast, retinoic acid had no effect on growth, or PKC expression in hormone independent, MDA-MB-231 breast cancer cells. By manipulating the expression of PKC α , we have shown that expression and activity of PKC α is sufficient to exert growth inhibitory effects on T-47D cells. The lack of retinoid effect on the hormone-independent MDA-MB-231 cell line could reflect the inability of retinoids to induce PKC α expression in these cells. To address whether this is the result of a defect in the PKC α gene, and to identify in more detail the role of PKC α in retinoid induced growth arrest of human breast carcinoma cells in terms of proto-oncogene expression, during year 2, we characterized the effect of expression of PKC α or antisense PKC α in MDA-MB-231 cells.

BODY FOR ANNUAL REPORT; YEAR 2.

Specific Aims.

Aim1. Determine if retinoic acid arrest of mammary carcinoma cells is associated with changes in the expression of, activation of, or signaling through the protein kinase C family (Task 1, 2 and 3).

Aim2. Determine the importance of PKC α and/or PKC ζ in mammary cell proliferation and/or differentiation (Task 4).

Task 1. Characterization of cell lines for expression, translocation and activity of PKC isozymes, month 1-10.

Methods

Cell culture

MDA-MB-231 human breast cancer cells (ATCC# HTB26) were grown on culture dishes in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum. Cells were treated with 10^{-6} M all trans retinoic acid dissolved in ethanol (final ethanol concentration was 0.1%) for up to 5 days or 100 ng/ml phorbol ester (TPA: phorbol 12-myristate 13-acetate) for 30 min prior to harvesting.

Measurement of relative proliferation

MDA-MB-231 cells were plated at 15×10^4 cells/60 mm culture dish. Media was changed 24 h later, at which time cells were treated with 10^{-6} M all trans retinoic acid dissolved in ethanol (final ethanol concentration was 0.1%). Media and experimental treatment were renewed every 72h. On the days indicated, cells were harvested, and counted with a hemocytometer. Each sample was counted in duplicate, and each condition was done in triplicate.

Measurement of colony forming

MDA-MB-231 cells were plated at 100 cells/60 mm culture dish. After 24 h all trans retinoic acid was added to a final concentration of 0 or 10^{-6} M from 1,000 x stock solutions dissolved in ethanol (final ethanol concentration was 0.1%). Media and experimental treatment were renewed every 72 h. After 21 days, cells were stained with 0.1 % Giemsa and colonies were quantified. Each sample was counted in triplicate, and each condition was done in six separate experiments.

Western blot analysis for PKC isozyme expression

Cell protein lysates were prepared and separated on 10% SDS-PAGE gel as described previously (Cho et al., 1997). Following electrophoretic transfer to nitrocellulose, membranes were blocked by 5% non fat dry milk in PBS. PKC isozymes were detected by incubating with PKC isozyme specific antibodies (polyclonal PKC α antibody; Transduction Lab., Lexington, KY.)(affinity purified polyclonal PKC β antibodies; protein G-purified polyclonal δ , ϵ and ζ antibodies)(Gibco BRL, Inc., Gaithersburg, MD), followed by extensive washing with PBS containing 0.05% Tween 20 (PBST) and subsequent incubation with the horseradish peroxidase coupled anti-rabbit IgG (1:7500 dilution)(Amersham Corp.). Immunoreactive proteins were visualized by enhanced chemiluminescence.

Subcellular fractionation

To fractionate subcellular compartments, cells were homogenized by polytron in ice cold 20 mM Tris-HCl buffer (pH 7.5) containing 2 mM EDTA, 0.5 mM EGTA, 250 mM sucrose, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μ g/ml leupeptin. The cell homogenates were clarified by low speed centrifugation (680g, 5min). Cleared supernatants was subjected to ultracentrifugation at 105,000g for 90 min to obtain the high- speed particulate(membrane) and the soluble (cytoplasmic) fractions. The particulate fraction obtained after ultra-centrifugation was solubilized in a homogenization buffer containing 0.2% NP-40 and sonicated for 3 second.

The particulate fraction obtained after low speed centrifugation (680g, 5 min), was further dissolved in 20 mM HEPES buffer (pH 7.0) containing 100 μ M vanadate, 100 μ M molybdate, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF and 0.2% NP-40. After low speed centrifugation (1500 g, 5 min) with 20 % glycerol and 400 mM NaCl, DNA and histone pellet is removed and the cleared supernatant was designated as nuclear fraction. The protein concentration of cytoplasmic, membrane and nuclear fractions was determined by Lowry et al (1951) using bovine serum albumin as a standard.

Assay of PKC activity

To measure total PKC activity, cells were lysed in 20mM Tris-HCl buffer (pH 7.5) containing 10mM EDTA, 1 mM EGTA, 0.5% NP40, and 0.4mM phenylmethylsulfonyl

fluoride for 10 min on ice. The cell lysates were clarified by centrifugation (12,000g, 15 min). The cleared supernatants were applied to DEAE-Sepharose columns (1 ml bed volume) for partial purification of total PKC protein. Columns were washed with 15 column volumes of 20mM Tris, 1 mM EDTA and 0.1 mM EGTA, and then PKC was eluted in 3 column volumes of the same buffer containing 90mM NaCl (preliminary experiments demonstrated that PKC activity eluted between 75 and 100 mM NaCl). The 90 mM NaCl eluates were assayed for PKC activity in 0.1 ml of 20 mM Tris-HCl (pH 7.5), 10mM MgCl₂, 0.2 mg/ml phosphatidylserine, 4 μ M diolein, 50 μ g/ml substrate, [γ -³²P]ATP (3x 10⁵ cpm/nmol, 10 μ M), and either 500 μ M CaCl₂ or 12 mM EGTA at 30°C for 10 min (Murray et al., 1993). Similar results were obtained using either histone H1, a modified pseudosubstrate site peptide, or a peptide corresponding to the myelin basic protein phosphorylation site as substrates. Reactions were stopped by spotting on phosphocellulose paper squares that were washed in 0.5% phosphoric acid before scintillation counting.

Results

In our first annual report, we reported that the inhibition of uncontrolled proliferation following retinoic acid treatment of hormone dependent, T-47D breast cancer cell lines, was consistent with retinoic acid inducing expression of PKC α and concomitant repression of PKC ζ expression. In contrast, retinoic acid had no effect on growth or PKC expression in hormone independent, MDA-MB-231 breast cancer cells. By manipulating the expression of PKC α , we further have shown that expression of PKC α is sufficient to exert growth inhibitory effects on T-47D cells. These findings suggest that PKC α plays an active role in mediating the anti-proliferative effect of retinoic acid in human breast cancer cells and also raised the question; Could the lack of retinoid effect on the MDA-MB-231 cells be explained by the inability of retinoids to induce PKC α expression in these cells? To address whether this is the result of a defect in the PKC α expression, we established MDA-MB-231 cells constitutively expressing PKC α or Antisense PKC α (As-PKC α) by retroviral infection of pMV7 vector encoding bovine PKC α (Borner et al., 1991) or As PKC α as described in methods (task 4).

Pools of G-418 resistant colonies were isolated and PKC activity was measured in extracts of transfected MDA-MB-231 cells which were treated with either RA (10⁻⁶ M to induced the growth arrest) or ethanol (as control) (Table 1). In G418 resistant control cells (pMV7 only), 100% of the total PKC activity was calcium independent, suggesting substantial expression of novel and atypical PKC isozymes, which are only diacylglycerol and/or phospholipid dependent. In our previous report (Cho et al., 1997), untreated MDA-MB-231 control cells expressed novel PKC (nPKC), PKC δ and ϵ , and atypical PKC ζ , but not conventional PKC (cPKC), PKC α , β . The absence of calcium dependent PKC activity (i.e. conventional PKC activity which is dependent on phospholipid, diacylglycerol and calcium) in G418 resistant control cells (pMV7 vector) confirmed the lack of expression of cPKC. RA treatment in G418 resistant control cells slightly increased the calcium dependent PKC activity (10.9 %) suggesting a trace of PKC α activity in these cells, which was barely detected by immunoblotting (lane 5 and 6 in Figure 1 and 2A). Anti-sense PKC α transfection abolished the slight increase of calcium dependent PKC activity in these cells. Calcium dependent PKC activity was largely increased (47.6%) as PKC α was transfected and further elevated (53.2%) with

retinoic acid treatment, suggesting the constitutive expression of PKC α in PKC α transfected MDA-MB-231 cells.

Protein extracts from transfected MDA-MB-231 cells were subjected to SDS-PAGE/Western blotting with PKC isozyme-specific antibodies. As expected from the kinase assays, control (C) or G418 resistant control cells (pMV7 vector, V) expressed only nPKC, PKC δ and ϵ , and atypical PKC ζ (Figure 1). No conventional PKC was detected. Retinoic acid (RA) treatment in MDA-MB-231 cells led no change of PKC isozyme expression. PKC α transfection resulted in constitutive expression of PKC α in control cells and allowed enhanced expression of PKC α by retinoic acid (RA) treatment (Figure 1). Despite the barely detectable level of endogenous PKC α protein (lane 5 and 6 in Figure 1 and 2A), this suggests the possibility that RA induces the expression of endogenous PKC α gene in MDA-MB-231 control cells, with a consistent view of PKC α as a transcriptionally response gene of RA via AP-2 promoter region (Haridas et al., 1998). In fact a trace of immunoreactive PKC α protein was measured (lane 5 in Figure 2A). Endogenous expression of other PKC isozymes such as PKC δ , PKC ϵ and PKC ζ was not altered in any of these cells (Figure 1).

To determine the subcellular distribution of PKC isozymes, transfected MDA-MB-231 cells were further fractionated into cytosolic, membrane and nuclear compartments and subjected to SDS-PAGE/Western blotting with PKC isozyme-specific antibodies. In PKC α expressing cells, PKC α proteins were equally distributed in cytosolic and membrane fraction (Figure 2 A and B). The expressed PKC α was also located in nuclear fraction (Figure 2C). Compared to a positive control of translocated PKC α expression in membrane (lane 9 in Figure 2B), RA treatment enhanced the translocation of PKC α into membrane fraction. In human breast cancer cells, PKC α desensitizes the epidermal growth factor receptor, EGFR, to the mitogenic effect of EGF, and as a result downstream signaling pathways are not activated, the AP-1 transcriptional factor is not synthesized or activated, and cells fail to progress through the cell cycle ((Cocekt et al., 1984; Friedman et al., 1984; Saloman, 1981; Lee and Weinstein 1978). Several lines of recent evidence also indicates that PKC α acts in the nucleus as well as the plasma membrane (Hyatt et al., 1990; Zauli et al., 1996; Yoshida et al., 1996; Schmalz et al., 1996). RA enhanced translocation of PKC α to membrane and nuclear fractions in PKC α expressing MDA-MB-231 cells suggests the possibility that PKC α desensitizes the growth factor receptor in membrane and combined with the possible transcriptional regulation of target gene in nucleus, ultimately mediates the regression of cell proliferation in response to RA treatment. The specific target of PKC α in membrane and nucleus is under investigation. Endogenous expression of other PKC isozymes such as PKC δ , ϵ and ζ was mainly located in cytosolic and membrane fraction (Figure 2A and 2B).

The above data confirmed the successful transfection of PKC α in MDA-MB-231 cells. As a first step toward investigating whether PKC α plays an active role in mediating the anti-proliferative effect of retinoic acid (RA) in human breast cancer cells, and to establish that the lack of RA effect on the MDA-MB-231 cells can be explained by the inability of RA to induce PKC α expression in these cells, we determined the proliferation of transfected MDA-MB-231 cells. Similar to previous reports (Cho et al., 1997), proliferation of control or G418 resistant control MDA-MB-231 cells (pMV7 vector) was insensitive to micromolar concentration of RA as shown in Figure 3A. Antisense PKC α transfected MDA-MB-231 cells were also insensitive to RA treatment.

Constitutive expression of PKC α in MDA-MB-231 cells itself did not inhibit proliferation (Figure 3A). However, with addition of 10^{-6} M RA, PKC α expressing MDA-MB-231 cells slightly reduced the proliferation after 3 days and was distinct after 5 days (40% reduction)(Figure 3B). To further determine the positive correlation of RA inability to induce PKC α expression and the lack of anti-proliferation effect in MDA-MB-231 cells, we performed the colony forming assay in transfected MDA-MB-231 cells (Table 2). With less cell plating (100 cells), anti-proliferation effect of PKC α was more distinct and resulted 22.67 % inhibition of cell proliferation. Similar to Figure 3B, proliferation of PKC α expressing MDA-MB-231 cells was further reduced (33.83% inhibition) with 10^{-6} M RA treatment for 5 days. Together, these data suggest that the lack of anti-proliferation effect of RA on MDA-MB-231 cells can be explained by the inability of RA to induce PKC α expression in these cells. In consistent view of anti-proliferation effect of retinoids in T-47D cells (Cho et al., 1997), retinoid arrested proliferation of MDA-MB-231 cells required the PKC α , presumably the retinoid-induced PKC α .

Task 2. Characterization of cell lines for proto-oncogene expression, month 11-16.

Methods

RNA isolation and northern blotting

To induce proto-oncogene expression, cells with >80% confluent were serum starved (0.5%) for 24hrs with or without RA (10^{-6} M) and then stimulated with 10% FBS (fetal bovine serum) for 30 min before harvesting. Total RNA was isolated from cells and northern blots were performed as described by Dorsett et al (1989). In every case, filters were hybridized to 32 P labeled RNA probes. Probes for L-30, c-fos, c-jun, JunB and JunD have been described previously (Talmage and Lackey, 1992). After washing in $2 \times$ SSC/1% SDS and $0.2 \times$ SSC/1% SDS, the northern blots were exposed to X-ray film at -80°C with intensifying screens.

Results

The above experiments identified that the inability of anti-proliferative effect of retinoic acid (RA) in MDA-MB-231 cells was associated with the lack of RA induced PKC α expression, suggesting that PKC α is an important mediator of the anti-proliferative effect of retinoic acid in human breast cancer cells. The mechanism(s) by which PKC α participates in anti-proliferation of MDA-MB-231 cells remains to be determined. The increase in steady state of PKC α increased the basal level of kinase activity leading to increased phosphorylation of cellular substrates that involves the regression of cell proliferation. Several recent reports demonstrated that PKC α phosphorylates and desensitizes the epidermal growth factor receptor (EGFR)(and/or similar receptor), and as a result downstream signaling pathways are not activated (Cocok et al., 1984; Friedman et al., 1984; Saloman, 1981; Lee and Weinstein 1978). Alternatively, the effects of PKC α might involve transcriptional regulation of the genes whose products are actively involved in removing MDA-MB-231 cells from the cell cycle. We determined and measured the serum induction of c-fos, c-jun, Jun B and JunD mRNA expression in MDA-MB-231 cells (Figure 4). Consistent with proliferation assays described in Figure 3 and Table 2, a 24 hour RA treatment of PKC α transfected MDA-MB-231 cells repressed the serum induction of c-fos expression and

concomitantly enhanced c-jun immediate early gene (IEG) induction (lane 15 in Figure 4).

Task 3. Characterization of retinoic acid effect on cell cycles progression, month 1-20.

Task 3 was not completed in first and second year. Over 2 years, we have been trying to establish T-47D stably expressing PKC α , PKC ζ , antisense PKC α or antisense PKC ζ or MDA-MB-231 cells stably expressing PKC ζ or antisense-PKC ζ . Unfortunately, all of transfected T-47D cells, or sense/antisense PKC ζ expressing MDA-MB-231 cells could not be survived. In third year, we are trying again to establish these transfected cell lines and plan to characterize intensively retinoic acid effect on cell cycle progression in each cell line including control T-47D and MDA-MB-231 cells.

Task 4. Manipulation of individual PKC isozyme gene expression, month 18-36.

Methods

Plasmids

The anti-sense PKC α plasmid (pMV7-As PKC α) was constructed by ligating the 2.44kb EcoR I fragment of PKC α cDNA from p β -actin SP72-PKC α (Cho et al., 1997) into the unique EcoR I site of the pMV 7 vector (Talmage and Lackey, 1992). The antisense-orientation was confirmed by BamH1 restriction digestion.

In Stratagene's LacSwitchTM II inducible mammalian expression system, sense or antisense PKC α cDNA (pOPRSVI-PKC α ; pOPRSVI-As PKC α) were subcloned by ligating the 2.46-kb Sma I and Kpn I fragment of PKC α cDNA from p β -actin SP72-PKC α into Sma I and Kpn I multicloning site of pOPRSVI/MCS. The sense or antisense orientation was confirmed by EcoR I.

Stable transfection

To stably express PKC α or antisense PKC α (As-PKC α), pSVX α encoding bovine PKC α (Borner et al., 1991) or pMV7-As PKC α was transfected into the NIH3T3 GP + env Am12 packaging cell line (Markowitz et al., 1988) and colonies stably producing recombinant virus were isolated following selection for G418 resistance (250 μ g/ml). MDA-MB-231 cells were infected with freshly conditioned medium derived from subconfluent NIH3T3 GP + env Am12 cultures. Following infections, cultures were subjected to G418 (250 μ g/ml) selection. Resistant cells were maintained in 100 μ g.ml⁻¹ G418.

For Stratagene's LacSwitchTM II inducible mammalian expression system, both pCMVLac I repressor and pOPRSVI/MCS encoding PKC α were transfected sequentially into MDA-MB-231 cells by lipofectAMINE reagent (Gibco BRL, Inc., Gaithersburg, MD)(Bebok et al., 1996). Following transfection, cells were subjected to hygromycin B (200 μ g/ml) and G418 (800 μ g/ml) selection over 4 weeks. Expanded colonies was isolated and treated with IPTG (isopropyl β -D-thiogalactopyranoside; 5 mM) for 6, 12, 18 and 24 hr followed by the IPTG removal for 48 or 72 hours to examine expression levels of PKC α induction and Lac repressor by western blotting with PKC antibody (polyclonal PKC α antibody; Transduction Lab., Lexington, KY.) and polyclonal antiserum to Lac repressor (Stratagene, Inc.)

Results

To delineate further the role of PKC α in RA induced growth arrest of human breast cancer cells, we used the Stratagene's Lac SwitchTM II inducible mammalian expression system, in which gene transcription can be reversibly turned on or off. In the escherichia coli lactose (lac) operon, the Lac repressor binds as a homotetramer to the lac operator, blocking transcription of the lacZ gene. Physiological or synthetic inducers, such as allolactose or isopropyl β -D-thiogalactopyranoside (IPTG), respectively, bind to the Lac repressor, causing a conformational change and effectively decreasing the affinity of the repressor for the operator. When the repressor is removed from the operator, transcription of genes from the lac operon resumes. In Stratagene's Lac SwitchTM II inducible mammalian expression system, sense/anti-sense PKC α cDNA was subcloned to pOPRSVI/MCS, in which RSV (Rous sarcoma virus)-LTR promoter drives expression of gene, and ideal operator sequences for the Lac repressor binding (op) are present in the RSV promoter and in the intron.

After sequential transfection of pLac and pOPR-PKC α in MDA-MB-231 cells, we examined the induction of PKC α expression by IPTG treatment in these cells. As shown in Figure 5A, the Lac repressor was constantly expressed as a dimer or monomer in all cells regardless of IPTG treatment. Compared to the positive control of PKC α expression in pOPR-PKC α single transfected MDA-MB-231 cells (lane 2 in Figure 5B), PKC α was not expressed initially in pLac + pOPR-PKC α transfected MDA-MB-231 cells (lane 4 in Figure 5B). By 6 hr IPTG treatment, PKC α expression was gradually induced and was maximal after 24 hr (Figure 5B). 48 hr after removing IPTG, PKC α had decreased to undetectable levels, confirming the reversibility of PKC α induction. For future experiments using pLac + pOPR-PKC α MDA-MB-231 cells, 24 hour IPTG treatment and 72 hour removal of IPTG was used for turning on/off of PKC α gene transcription.

The serum induction of c-fos and c-jun expression in PKC α expressed MDA-MB-231 cells by IPTG treatment was determined as shown in Figure 6. As a control, we also measured c-fos expression in control, and pLac or pOPR-PKC α single transfected MDA-MB-231 cells (Figure 6A). As shown earlier in cells constitutively expressing PKC α (Figure 4) or transfected pOPR-PKC α only (lane 12 in Figure 6A), concomitant 24 hr RA and IPTG treatment of pLac and pOPR-PKC α transfected cells repressed c-fos expression (lane 8 in Figure 6B). This effect was lost 72 hour after removing out the IPTG (lane 12 in Figure 6B). As soon above, c-jun expression was enhanced and then repressed. In Figure 4 and 6, PKC α expressing MDA-MB-231 cells showed a response to retinoic acid in terms of modulating c-fos and c-jun expression. These cells also showed the regression of cell proliferation in the presence of RA (Figure 3 and Table 2).

CONCLUSION

The data presented in this annual report support our hypothesis for a mechanism of retinoic acid (RA)-induced growth inhibition of human breast cancer cells. The induction of PKC α expression following RA treatment coincided with the cessation of proliferation of hormone dependent, T-47D breast cancer cells. In contrast, RA had no effect on growth or PKC expression in hormone-independent MDA-MB-231 cells. To determine whether the lack of RA effect on MDA-MB-231 cells is caused by the failure

of PKC α expression, we established MDA-MB-231 cell lines stably expressing PKC α or antisense PKC α RNAs. MDA-MB-231 cells transfected with PKC α , constitutively expressed PKC α in cell cytosol, membrane and nucleus. RA treatment enhanced cPKC activity and the translocation of PKC α into the cellular membrane and nucleus. Although constitutive expression of PKC α had no effect by itself, on MDA-MB-231 proliferation, it allowed RA to inhibit both serum induction of c-fos expression and cell proliferation (by ~40%). By turning on/off the transcription of PKC α gene, we further have shown the PKC α regulation of c-fos and c-jun expression as a downstream target. In total, our data demonstrate that the lack of anti-proliferation effect of RA in MDA-MB-231 cells is partially caused by the failure of RA to induce PKC α expression and consequently the absence of PKC α altered gene expression.

It is not clear how PKC α expression changed MDA-MB-231 cell sensitivity to RA. Several observations strongly favor RAR α as the mediator of RA growth regulation and PKC α expression in T-47D cells (Cho et al., 1997; Sheikh et al., 1994; Valette et al., 1987; Kennedy et al., 1992). For example, RAR α is the major RAR subtype expressed in T-47D cells and the synthetic retinoid Am580 arrests T-47D proliferation at concentration ($<10^{-8}$ M), at which it demonstrates greatest selectivity for RAR α (Delescluse et al., 1991). RAR α is present at very low levels, and RAR γ is the major RAR subtype in MDA-MB-231 cells. (Sheikh et al., 1994). The lack of RA effect on MDA-MB-231 cell proliferation or PKC α expression could reflect the relative lack of RAR α . Constitutive expression of PKC α repaired the RA inability for RAR α dependent induction of PKC α expression (Figure 1 and 2) and RA treatment can increase the level of RAR γ expression (Sheikh et al., 1994). By prolonged treatment (5 days) of micromolar concentration of RA, constitutively expressed PKC α in MDA-MB-231 cells can phosphorylate RAR γ which expression is increased with RA treatment. Although activated RA-RAR γ and PKC α synergistically affect transcriptional regulation of c-fos and c-jun and ultimately slowed MDA-MB-231 cell progress through cell cycle, the effect was not equivalent to the strong and rapid growth suppression of T-47D cells by retinoic acid. MDA-MB-231 cells expressing a transfected RAR α gene also are considerably less responsive to retinoic acid than T-47D cells (Sheikh et al., 1994). Based on our findings, cotransfection of PKC α and RAR α in MDA-MB231 cells might result to increase the RA sensitivity comparable to hormone dependent T-47D cells.

Future work: we have described an important role of PKC α in mediating the anti-proliferative action of retinoic acid in human breast cancer cells. Our goal is to identifying in more detail the role of PKC α in retinoid induced growth arrest of human breast cancer cells in terms of proto- oncogene and cell cycle progression. During first and second year of this project, we only established the stable transfection of sense/antisense PKC α in MDA-MB-231 cells. After stable transfection of sense/antisense PKC α in T-47D and sense/antisense PKC ζ in T-47D and MDA-MB-231 cells, we plan to complete Task 2 in transfected T-47D cells, and Task 3 in transfected T-47D and MDA-MB-231 cells.

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Appendix

Table 1-2, Figure 1-6, Attached.

At the end of Figures, publication and meeting abstract produced during September 1997-September 1998 was attached.

Figure legends

Figure 1. Protein Kinase C isozyme expression in transfected MDA-MB-231 cells.

Whole Cell extracts (30 μ g protein/lane) from control (C; lanes 1, 5), G418 resistant control (pMV7 vector only, V; lanes 2, 6), PKC α (α ; lanes 3, 7) or antisense PKC α (As α ; lanes 4, 8) transfected MDA-MB-231 cells were subjected to SDS-PAGE and western blotting with PKC isozyme specific antibodies (polyclonal PKC α antibody; Transduction Lab., Inc., Lexington, KY.) (affinity purified polyclonal PKC β antibody; protein G-purified polyclonal PKC δ , ϵ and ζ antibodies) (Gibco BRL, Inc., Gaithersburg, MD). MDA-MB-231 cells in lanes 5-8 were treated with retinoic acid (RA) (10^{-6} M) for 5 days. The region of each gel shown was between the Mr 67,000 and 93,000 prestained molecular weight markers (New England Biolabs, Beverly, MA) run in adjacent lanes.

Figure 2. Subcellular distribution of PKC isozyme in transfected MDA-MB-231 cells.

To determine the subcellular distribution of PKC isozyme, cytosolic, membrane or nuclear protein extracts (15 μ g protein /lane) from control (C; lanes 1, 5), G418 resistant control (pMV7 vector only, V; lanes 2, 6), PKC α (α ; lanes 3, 7) or antisense PKC α (As α ; lanes 4, 8) transfected MDA-MB-231 cells were subjected to SDS-PAGE followed by western blotting with PKC isozyme specific antibodies as described under methods. MDA-MB-231 cells in Lanes 5-8 were treated with retinoic acid (RA) (10^{-6} M) for 5 days. As a positive control of translocated PKC α expression in membrane fraction, cytosolic, membrane or nuclear protein extracts (7.5 μ g protein /lane) from PKC α transfected MDA-MB-231 cell followed by 30 min stimulation of 100ng/ml phorbol ester(+TPA) was loaded in lane 9. The region of each gel shown was between the Mr 67,000 and 93,000 prestained molecular weight markers (New England Biolabs, Beverly, MA) run in adjacent lanes.

Figure 3. Retinoic acid becomes to inhibit proliferation of MDA-MB-231 cells with constitutive expression of PKC α .

Hormone independent control, G418 resistant control (pMV7 vector only), PKC α or antisense PKC α transfected MDA-MB-231 cells were plated at 15×10^4 cells/dish. After 24hr retinoic acid was added to a final concentration of 0 or 10^{-6} M from 1,000x stock solutions in ethanol. Media and experimental treatment were renewed every 72 hrs. Cells were trypsinized, stained with trypan blue, and counted after indicated intervals. Data shown (\pm SE) are from three independent experiments in which treatments were done in duplicate. Day 1 represents the time of retinoid or solvent addition.

Figure 4. Stable expression of PKC α alters proto-oncogene expression.

Control (C), G418 resistant control (pMV7 vector only, V), PKC α (α) or antisense PKC α (As α) transfected MDA-MB-231 cells were maintained for 24 hrs in either 10% fetal bovine serum (FBS), 0.5% FBS (Serum starvation) or 0.5% FBS + retinoic acid (RA; 10^{-6}

M). For induction of proto-oncogene expression, 0.5% FBS or 0.5% FBS + 10^{-6} M RA treated MDA-MB-231 cells were stimulated with 10% FBS for 30 min before harvesting. Total RNA was isolated and analyzed by northern blotting for expression of L-30, c-fos, c-jun, JunB and JunD.

Figure 5. IPTG Induced PKC α expression in Lac repressor and PKC α cotransfected MDA-MB-231 cells.

The expanded clone #4 of pCMVLac 1 repressor (pLac) and pOPRSVI/MCS-PKC α (pOPR α) cotransfected MDA-MB-231 cells were treated with IPTG for 6, 12, 18 or 24 hrs followed by IPTG removal for 48 hrs or 72 hrs. Whole cell extracts (15 μ g protein /lane) were subjected to SDS-PAGE and western blotting with polyclonal antiserum to Lac repressor (Stratagene, Inc.)(A) or PKC α antibody (polyclonal PKC α antibody; Transduction Lab., Inc., Lexington, KY)(B). For a positive control of either Lac repressor expression or PKC α expression, whole cell extracts (15 μ g protein /lane) of single transfected MDA-MB-231 cells with pLac I repressor or pOPR α was loaded in lane 1 or 2. Also, whole cell extracts (15 μ g protein /lane) from OPRSVI/MCS-AntisensePKC α (pOPR-As α) transfected MDA-MB-231 cells was loaded in lane 3.

Figure 6. PKC α expressed MDA-MB-231 cells by IPTG have altered c-fos and c-jun expression.

A. Control (C), pCMVLac I repressor (pLac) or pOPRSVI/MCS-PKC α (pOPR α) single transfected MDA-MB-231 cells were maintained for 24 hrs in either 10% fetal bovine serum (FBS), 0.5% FBS (Serum starvation) or 0.5% FBS + retinoic acid (RA; 10^{-6} M). For induction of proto-oncogene expression, 0.5% FBS or 0.5% FBS + 10^{-6} M RA treated MDA-MB-231 cells were stimulated with 10% FBS for 30 min before harvesting. Total RNA was isolated and analyzed by northern blotting for expression of L-30, c-fos and c-jun. **B.** In parallel with serum starvation/stimulation treatment in Figure 6A, the expanded clone #4 of pLac and pOPR α cotransfected MDA-MB-231 cells were further treated with IPTG for 24hr followed by IPTG removal for 72 hrs for turning on/off of PKC α transcription. Total RNA was isolated and analyzed by northern blotting for expression of L-30, c-fos and c-jun.

Table 1. Protein Kinase C activity in transfected MDA-MB231 cells

Transfected MDA-MB-231 cells	PKC activity (10 ⁶ cpm/min/mg protein)			% Calcium dependency
	Cofactors in Assay			
	DAG/PS/Ca ⁺⁺	DAG/PS	PS	
pMV7	1.1 ± 0.11	1.14 ± 0.11	0.37 ± 0.05	-
PKC α	2.29 ± 0.30	1.2 ± 0.14	ND	47.6
AsPKC α	1.18 ± 0.16	1.6 ± 0.31	0.34 ± 0.16	-
pMV7 + RA	1.92 ± 0.16	1.71 ± 0.55	0.25 ± 0.10	10.9
PKC α + RA	3.55 ± 0.65	1.66 ± 0.45	ND	53.2
AsPKC α +RA	1.86 ± 0.61	1.8 ± 0.26	0.27 ± 0.13	-

G418 resistant control (pMV7 vector only), PKC α (PKC α) or antisense PKC α (As-PKC α) transfected MDA-MB-231 cells were further treated with 10⁻⁶ M retinoic acid (RA) for 5 days. The detergent extract were absorbed to DEAE-Sepharose, and the fraction eluting with 90 mM NaCl was assayed for PKC activity in the presence of diacylglycerol (DAG)/phosphatidylserine (PS)/Ca⁺⁺, DAG/PS/EGTA (-calcium) or PS only. Activity (\pm SE) is shown as ³²P transferred from ATP to peptide substrate/min/mg extract protein at 30°C. The increase in calcium dependent activity with PKC α transfection and 5 day RA treatment were significant at p< 0.05 (as determined using Student's t-test).

Table 2. Inhibited colony formation of MDA-MB231 cells with constitutive expression of PKC α and Retinoic Acid (RA) treatment.

MDA-MB231 cells	1	2	3	4	5	6	mean (\pm SE)	p value	% inhibition
control	37	39	51	50	50	53	46.7(2.8)		0
pMV7	40	36	52	52	51	50	46.8(2.9)		0
PKC α	29	28	44	40	38	38	36.2(2.6)	.003	22.67
As-PKC α	33	38	48	53	60	52	47.3(4.1)		0
control + RA	40	ND	ND	50	50	54	48.5(3.0)		0
pMV7 + RA	37	ND	ND	51	50	50	47.0(3.3)		0
PKC α + RA	23	25	36	31	37	34	31.0(2.4)	.005	33.83
As-PKC α + RA	43	39	50	48	52	55	47.8(7.4)		0

Control, G418 resistant control (pMV7 vector only) MDA-MB-231 cells, PKC α (PKC α) or antisense PKC α (As-PKC α) transfected MDA-MB-231 cells were plated at 100 cells/dish. After 24hr retinoic acid was added to a final concentration of 0 or 10^{-6} M from 1,000x stock solutions in ethanol. Media and experimental treatment were renewed every 72 hrs. After 21 days, cells were stained with 0.1% Giemsa and colonies were counted. The results from six separate experiments with triplicate are shown as average colonies per 60-mm dish. The percent of inhibition was determined by using the formula: $1 - (\text{colony\# in each cell line} / \text{colony \# in control cell line})$. The inhibited colony formation with constitutive expression of PKC α ($P = 0.0003$) and constitutive expression of PKC α + retinoic acid (RA) treatment ($P = 0.005$) were significant at $P < 0.05$ vs. control (as determined using student's t-test). The average percent of inhibition by PKC α transfection or PKC α transfection + 10^{-6} M RA is shown in the last column. ND, not determined.

Figure 1

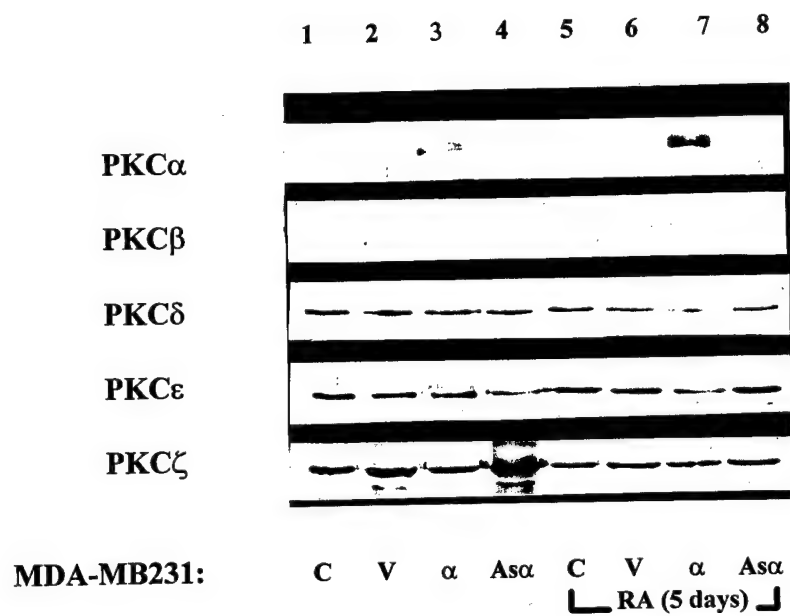
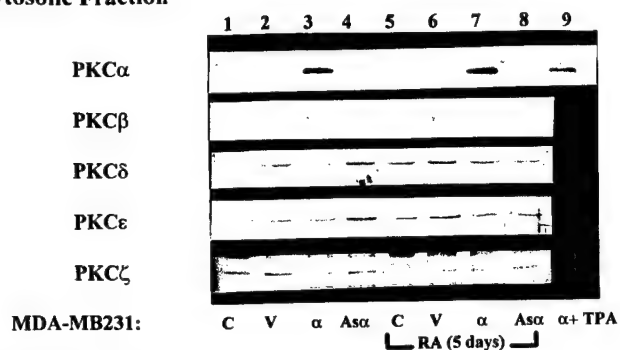
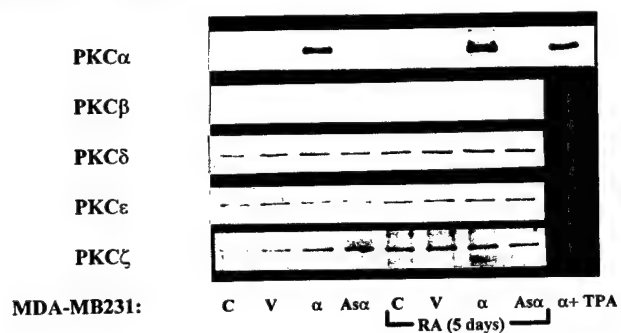


Figure 2.

A. Cytosolic Fraction



B. Membrane Fraction



C. Nuclear Fraction

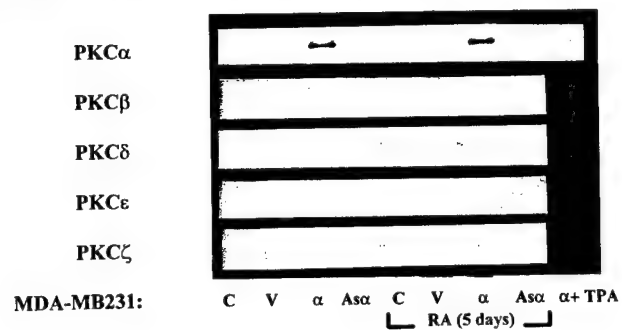


Figure 3

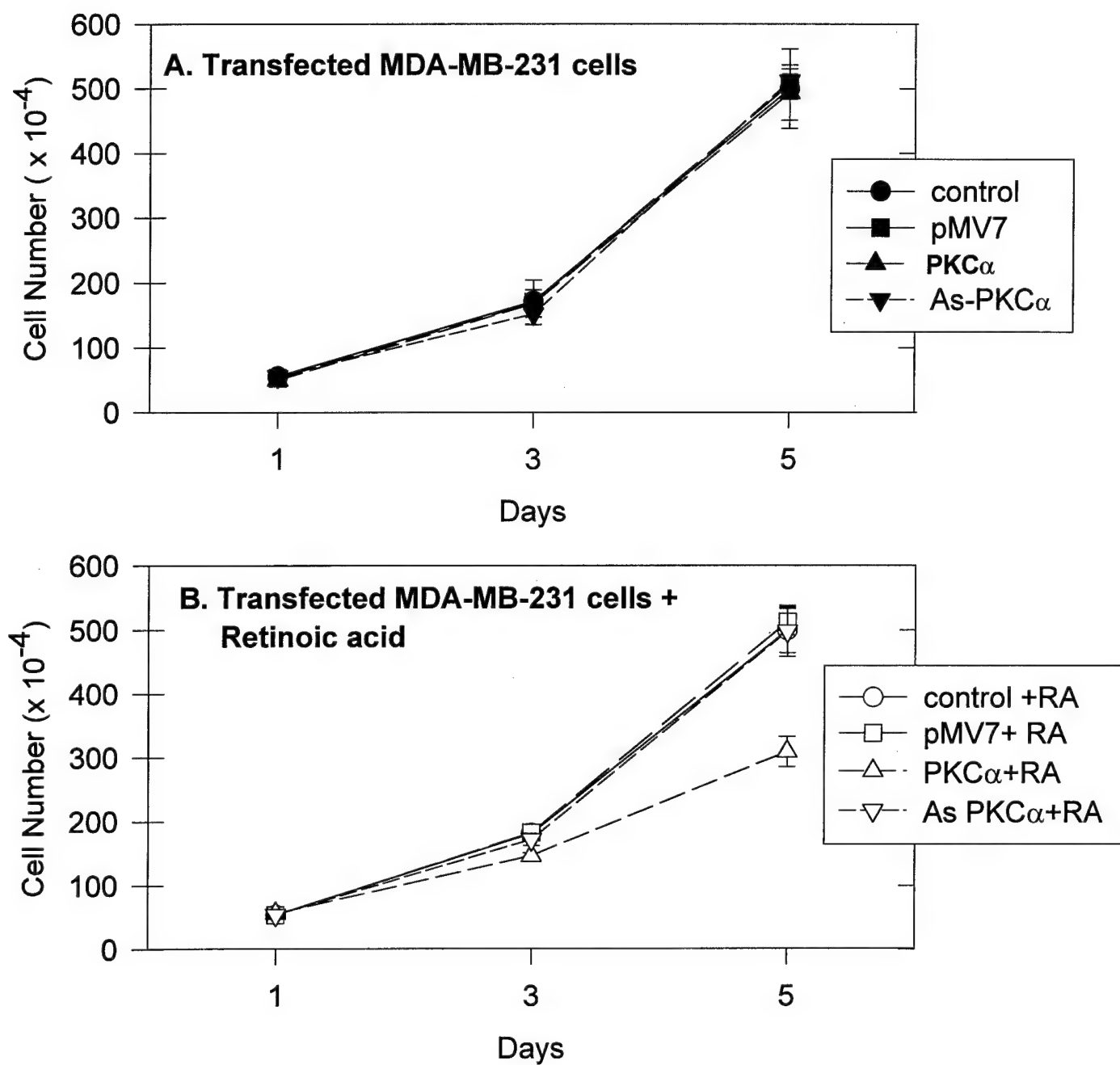


Figure 4.

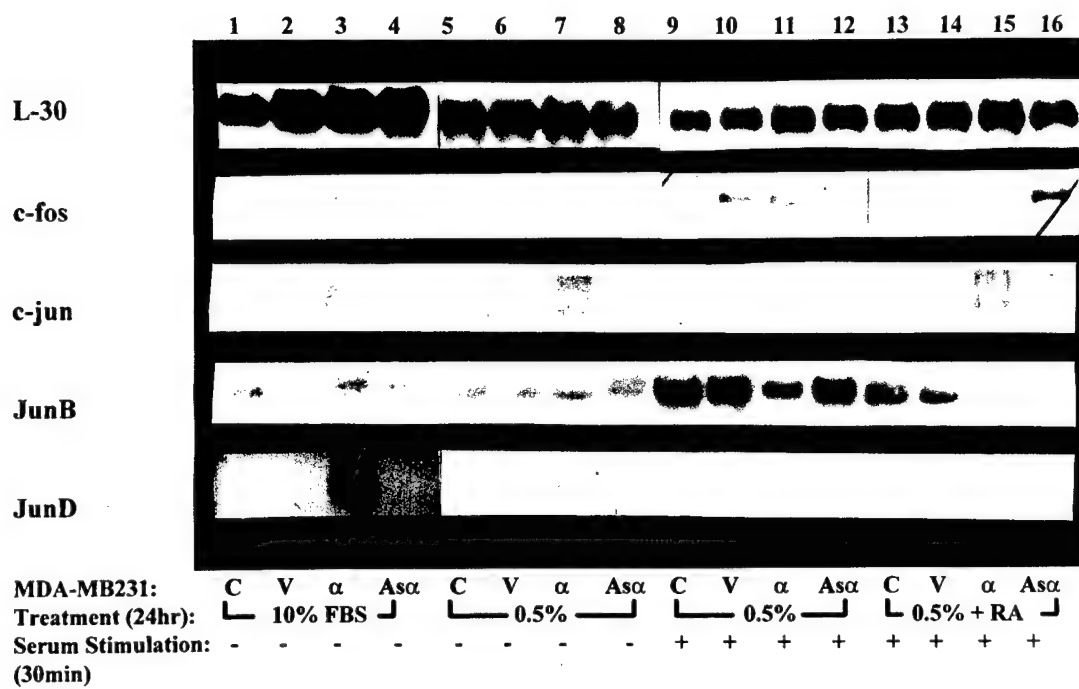


Figure 5.

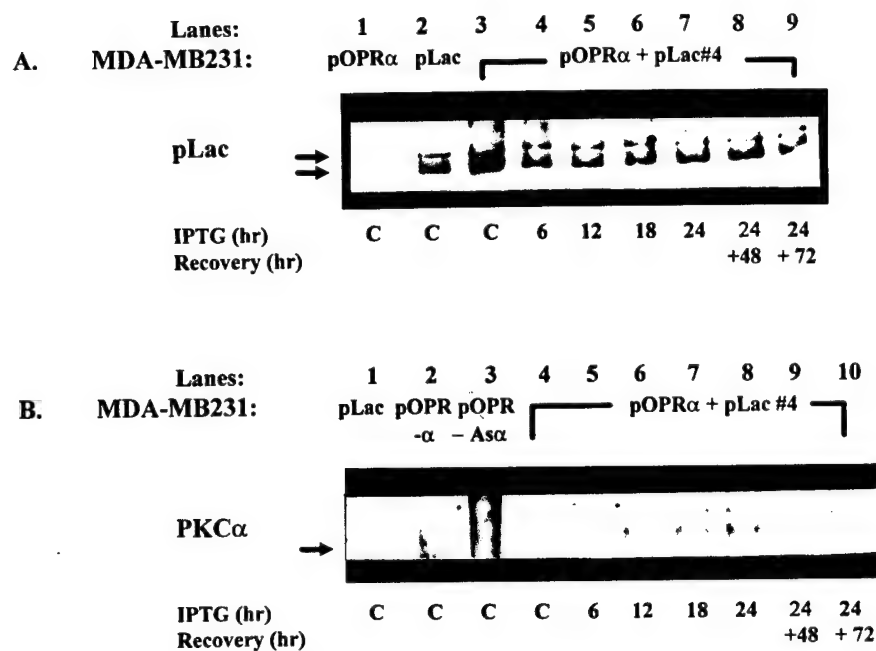
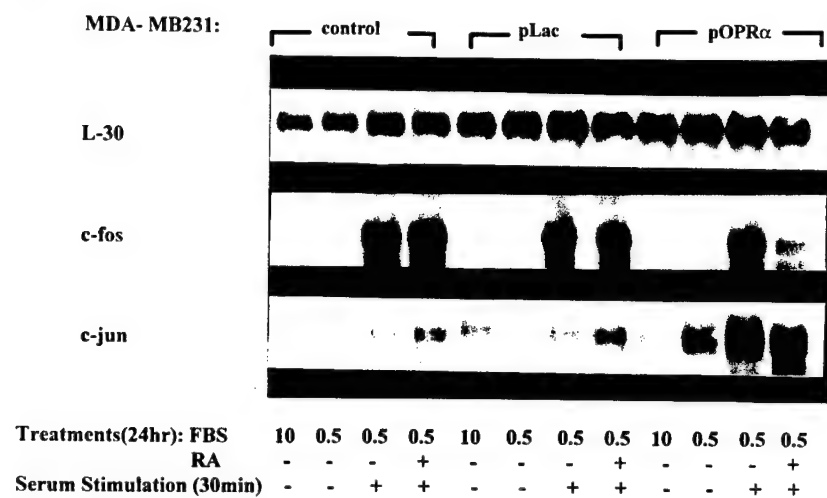
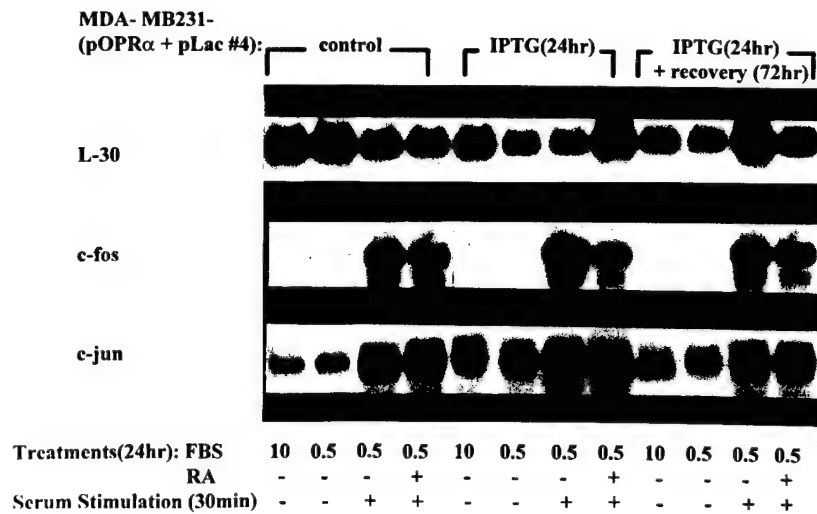


Figure 6

A.



B.



Bibliography

Meeting Abstract

Retinoids'97; European Retinoid Research Group
(September 28 – October 1, 1997; Nice, France)

Abstract # A099

Retinoic acid –induced cell cycle arrest of a human breast cancer cell line is PKC α -dependent.

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We hypothesize that retinoids inhibit hormone-dependent human breast cancer cell proliferation by inducing a state of functional mitogen deprivation. Retinoic acid reversibly arrested proliferation of hormone-dependent breast cancer cells. Retinoic acid treatment repressed epidermal growth factor induction of genes involved in progression through the G₁ phase of the cell cycle, leading to accumulation of cells in G₀/G₁. Retinoic acid treatment inhibited all measures of epidermal growth factor signaling including: (1) epidermal growth factor receptor kinase activity, (2) activation of p70^{s6k}, Jun-N-terminal kinase, p38 kinase and mitogen activated protein kinase, and (3) immediate early gene expression. We have previously shown retinoic acid-induced expression of protein kinase C α . The antagonistic effect of retinoic acid on epidermal growth factor signaling is prevented by inhibiting protein kinase C α activity. We conclude that retinoic acid arrests cell cycle progression by first inducing protein kinase C α expression. Subsequent activation of protein kinase C α inhibits mitogenic signal transduction from the epidermal growth factor receptor.

Distinct Functions of Protein Kinase C α and Protein Kinase C β during Retinoic Acid-induced Differentiation of F9 Cells¹

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Abstract

As F9 embryonal carcinoma cells differentiate into parietal endoderm-like cells, expression of conventional protein kinase C (PKC) changes. Undifferentiated stem cells express PKC β but not PKC α , whereas differentiated parietal endoderm cells express PKC α but not PKC β . To determine whether changes in PKC α and/or PKC β expression control retinoic acid (RA)- and dibutyryl cyclic AMP-induced F9 cell differentiation, we established cell lines stably expressing PKC α , PKC β , antisense PKC α , or antisense PKC β RNAs. Constitutive expression of PKC α or inhibition of PKC β expression in F9 stem cells enhanced RA induced differentiation, both by increasing total expression and accelerating RA-induced expression of laminins A, B1, B2, and type IV collagen. In addition, expressing PKC β in a parietal endoderm cell line caused these cells to retrodifferentiate into stem cells. Based on these results, we conclude that PKC β and PKC α are key targets for RA-regulated gene expression, that PKC α plays an important, active role in inducing and maintaining the parietal endoderm phenotype, and that PKC β activity is incompatible with maintaining the differentiated state of these cells.

Introduction

F9 mouse embryonal carcinoma cells have limited developmental potential and exhibit little spontaneous differentiation. When treated with RA,³ F9 cells differentiate into primitive endoderm-like cells (1, 2), which have the ability to respond to secondary differentiation factors that determine the ulti-

mate differentiated phenotype. Exposure of these primitive endoderm-like cells to agents that elevate intracellular cAMP induces formation of parietal endoderm (1, 2). In the absence of cAMP, aggregates of RA-treated F9 cells differentiate into a cell type resembling the visceral endoderm of the mouse embryo (3, 4).

During F9 differentiation, RA profoundly alters the expression of intracellular signaling pathways. Notable among the reported changes are increases in the AP-1 transcription factor (5-9), increased total PKC activity (10-12), and increased levels of phorbol ester receptors (13). RA also increases the amount and alters the subcellular distribution of protein kinase A, changes that are believed to contribute to the ability of cAMP to induce parietal endoderm differentiation (14).

PKC is a calcium-, diacylglycerol-, and phospholipid-dependent enzyme involved in regulating differentiation and proliferation (15, 16). There are at least 11 distinct genes constituting the PKC family that can be grouped into three subfamilies based on sequence homology and cofactor requirement: the conventional PKCs (α , β 1, β 2, and γ) are calcium, diacylglycerol, and phospholipid dependent; the novel PKCs (δ , ϵ , θ , η , and μ) are diacylglycerol and phospholipid dependent but calcium independent, whereas the atypical PKCs (λ and ζ) are phospholipid dependent but diacylglycerol and calcium independent (16). Tumor-promoting phorbol esters are potent diacylglycerol agonists that bind to and activate both conventional PKCs and novel PKCs but not atypical PKCs (17, 18). In addition to activating these enzymes, chronic treatment of cells with phorbol esters down-regulates conventional PKCs and novel PKCs (16).

The increased level of calcium-dependent PKC activity reported during RA- and cAMP-induced differentiation of F9 cells into parietal endoderm results from increased expression of PKC α (11, 12); concurrently, PKC β expression declines. F9 stem cells and visceral endoderm cells express PKC β but not PKC α , whereas F9 cells differentiating into parietal endoderm express PKC α but not PKC β . The transition from PKC β to PKC α is accompanied by changes in phorbol ester-induced gene expression. Specifically, phorbol ester induces c-fos expression in PKC β -expressing F9 stem cells but not in PKC α -expressing parietal endoderm cells. In contrast, phorbol ester induces type IV collagen, a parietal endoderm marker gene, in PKC α -expressing parietal endoderm cells.

To establish a functional role for PKC α and PKC β in mediating parietal endoderm differentiation, we established F9-derived cell lines constitutively expressing PKC α , PKC β , antisense PKC α , or antisense PKC β RNAs. Constitutive expression of PKC α in stem cells was sufficient to allow phorbol ester induction of type IV collagen even in the absence of

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³ The abbreviations used are: RA, retinoic acid; cAMP, cyclic AMP; PKC, protein kinase C; TTR, transthyretin; TPA, 12-O-tetradecanoylphorbol-13-acetate; dbcAMP, dibutyryl cAMP.

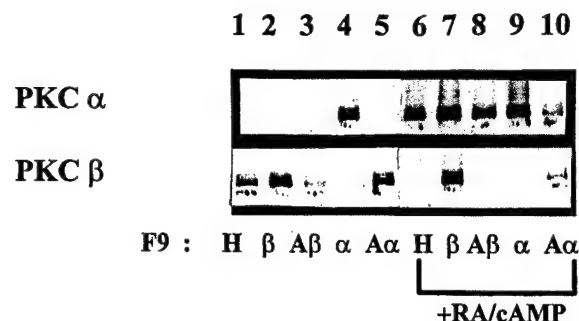


Fig. 1. PKC α and PKC β expression in transfected F9 cells. Cell extracts (30 μ g protein/lane) from hygromycin-resistant control F9 cells (H, Lanes 1 and 6), PKC β (β , Lanes 2 and 7), antisense PKC β (A β , Lanes 3 and 8), PKC α (α , Lanes 4 and 9), and antisense PKC α (A α , Lanes 5 and 10) transfected F9 cells were subjected to SDS-PAGE and Western blotting with either PKC α - or PKC β -specific antibodies (affinity-purified polyclonal PKC α and PKC β antibodies; Life Technologies, Inc.). F9 cells in Lanes 6–10 were treated with RA (2×10^{-7} M) and dbcAMP (5×10^{-4} M) for 5 days. The region of each gel shown was between the M_r 67,000 and 93,000 prestained molecular weight markers run in adjacent lanes.

RA and cAMP. Furthermore, PKC α expression both accelerated and increased the total expression of laminin A/B1/B2 and type IV collagen in response to RA and cAMP. Antisense PKC α or PKC β expression decreased RA and cAMP induction of these parietal endoderm markers and alternatively enhanced the expression of visceral endoderm marker genes. In addition, expressing PKC β in a parietal endoderm cell line gradually caused these cells to retrodifferentiate into stem cells. These results indicate that PKC α is critical in establishing and maintaining the parietal endoderm phenotype, and furthermore, that PKC β is incompatible with maintaining the differentiated state of these cells. PKC β expression is associated with maintaining stem cell proliferation and inducing visceral endoderm formation.

Results

Constitutive Expression of PKC β and PKC α after Stable Transfection of F9 Cells. F9 cells were cotransfected with a plasmid encoding hygromycin resistance and expression plasmids encoding either PKC β , antisense PKC β , PKC α , or antisense PKC α cDNA. Expression in each case was controlled by the β -actin promoter, which is expressed in both differentiated and undifferentiated F9 cells (11). Pools of hygromycin B-resistant colonies were isolated. As shown in Fig. 1 and Table 1, when compared with F9 stem cells expressing only the *hph* gene (H; conferring hygromycin B resistance), expression of PKC β from the β -actin promoter elevated total PKC β levels in stem cells by 2.2-fold and allowed continued PKC β expression following RA + cAMP treatment. Expression of antisense PKC β RNA decreased steady-state PKC β levels in F9 stem cells by 50% (Fig. 1, Lane 3). Transfection with the PKC α -expressing plasmid resulted in the constitutive expression of PKC α in stem cells. Induction of the endogenous PKC α gene by RA + cAMP was unaffected in cells transfected with PKC β , antisense PKC β , or PKC α . Constitutive expression of antisense PKC α RNA partially prevented RA + cAMP-induced PKC α expression

Table 1 Relative levels of PKC α and PKC β expression in transfected F9 cells

Films from Fig. 1 were scanned with a densitometer, and the area of the resulting peaks were integrated. For PKC α , the fold induction was calculated over the area value found in RA + cAMP-treated, hygromycin-resistant F9 cells (Lane 6). For PKC β , the fold induction was calculated over the area value of hygromycin resistant F9 control cells (H; Lane 1). Each value is indicated as fold unit.

	H	β	A β	α	A α	RA + cAMP				
						H	β	A β	α	A α
PKC α	ND ^a	ND	ND	1.4	ND	1.0	1.7	0.9	1.8	0.3
PKC β	1.0	2.2	0.5	ND	1.8	ND	2.7	ND	ND	0.5

^a ND, not detected.

(by 70%). Endogenous expression of other PKC isozymes such as PKC γ , δ , ϵ , and ζ was not altered in any of these cells (data not shown). As we reported previously (11), differentiation-induced changes in PKC β and PKC α reflect altered mRNA levels (data not shown). Experimental manipulation of PKC β (either up or down) had little effect on PKC α expression (mRNA or protein). In contrast, expression of PKC α in F9 stem cells decreased PKC β expression. F9 stem cells transfected with the PKC α plasmid had no detectable PKC β (Fig. 1, Lane 4). The negative effect of PKC α on PKC β expression was confirmed in cells expressing antisense PKC α RNA. When these cells were induced to differentiate with RA + cAMP, PKC α expression remained low, and the loss of PKC β expression was incomplete (Fig. 1, Lane 10). In contrast, constitutive expression of PKC β did not prevent PKC α expression in differentiating F9 cells (Fig. 1, Lane 7).

PKC β Enhanced c-fos Expression and PKC α Allowed Phorbol Ester-activated Expression of Collagen IV in F9 Stem Cells. The transition from PKC β to PKC α in differentiating F9 cells is accompanied by changes in phorbol ester stimulation of target gene expression (11). Specifically, phorbol ester induced c-fos expression in PKC β -expressing stem cells but not in PKC α -expressing parietal endoderm cells. In contrast, phorbol ester induced type IV collagen, a parietal endoderm marker gene, in F9 stem cells transfected with and expressing PKC α . The causal relationship between PKC β activity and c-fos expression and between PKC α activity and collagen IV expression was supported by altering expression of PKC β or PKC α in F9 cells independently of RA + cAMP treatment (Fig. 2 and Table 2). PKC β expression increased basal expression of both c-fos and c-jun to levels equal to (c-fos) or greater than (c-jun) seen following phorbol ester treatment of F9 stem cells (Table 2; hybridization intensities in each sample were normalized to the L-30 ribosomal protein mRNA control). Decreasing PKC β by expressing antisense PKC β RNA had the opposite effect; decreasing both basal and phorbol ester induced expression of c-fos and c-jun. Changes in c-fos/c-jun expression resulted specifically from changes in PKC β and did not simply reflect increased total PKC activity (11). Expression of comparable levels of PKC α caused little or no alteration of basal c-fos and c-jun expression in F9 stem cells (Fig. 2, Lane 4). Although these results support a link between PKC β activity and c-fos expression in stem cells, constitutive expression of

Fig. 2. PKC α/β -expressing F9 cells have altered basal and phorbol ester-induced c-fos, c-jun, and type IV collagen expression. Total RNA (20 μ g/lane) from hygromycin-resistant control (H, Lanes 1 and 6), PKC β (β , Lanes 2 and 7), antisense PKC β (β , Lanes 3 and 8), PKC α (α , Lanes 4 and 9), and antisense PKC α (α , Lanes 5 and 10) transfected F9 cells was analyzed using Northern blotting for expression of c-fos, type IV collagen, c-jun, and L-30. Cells in Lanes 6–10 were treated with RA (2×10^{-7} M) and dbcAMP (5×10^{-4} M) for 5 days. One h before harvesting, one-half of the cells were stimulated with 100 ng/ml phorbol ester (+TPA, lower panel). The Northern blots were exposed to X-ray film for 3 days (c-fos, c-jun, and collagen) or overnight (L-30) at -80°C . L-30 is mRNA expression control.

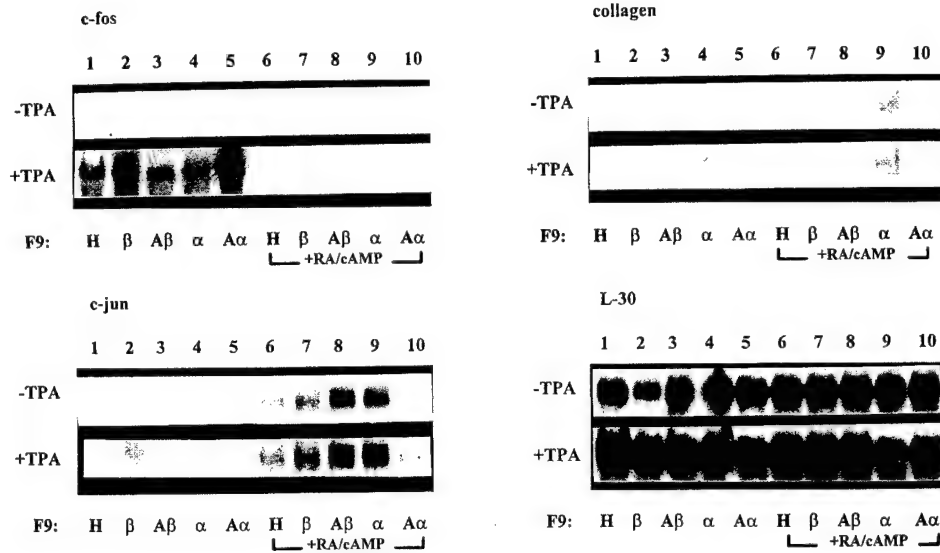


Table 2 Relative levels of c-fos, c-jun, and type IV collagen mRNA expression in transfected F9 cells

Films from Fig. 2 were scanned with a densitometer, and the integrated area of the resulting peaks were normalized to the integrated area of the corresponding L-30 peak. For c-fos and c-jun mRNA levels, the fold induction of each treated cells was calculated over the area value found in hygromycin-resistant F9 control cells (H; Lane 1). For type IV collagen mRNA levels, the fold induction was calculated over the area value of RA + cAMP-treated, hygromycin-resistant F9 control cells (Lane 6).

A. c-fos										
	H	β	A β	α	A α	RA + cAMP				
						H	β	A β	α	A α
c-fos										
-TPA	1.0	4.2	ND ^a	ND	ND	ND	ND	ND	ND	ND
+TPA	4.3	8.0	2.8	2.3	7.4	ND	ND	ND	ND	ND
B. c-jun										
c-jun										
-TPA	1.0	6.7	0.2	1.0	0.4	4.8	13.8	25.8	20.7	1.9
+TPA	1.7	8.2	0.6	2.7	2.6	5.7	17.0	27.6	34.3	4.8
C. collagen										
Type IV collagen										
-TPA	ND	ND	ND	ND	ND	1.0	0.3	2.4	3.4	0.2
+TPA	ND	ND	ND	1.1	ND	0.4	0.2	0.5	2.9	0.2

^a ND, not detected.

PKC β in RA + cAMP-treated F9 cells did not restore c-fos expression in response to phorbol ester (Fig. 2, Lane 7).

Steady-state c-jun mRNA levels were ~5-fold higher in RA + cAMP-treated, hygromycin-resistant cells than in stem cells (Fig. 2, Lane 1 versus Lane 6; Table 2). Although phorbol ester treatment had only a modest effect on c-jun expression, increasing PKC β expression resulted in elevated c-jun mRNA, both in stem cells and in differentiated cells (Fig. 2, Lanes 2 and 7; Table 2, 8.2- and 17-fold). PKC α expression in stem cells had no effect on c-jun expression but was associated with substantially increased c-jun expression in RA + cAMP-treated cells (Table 2, 20.7-fold), an effect reversed by antisense PKC α RNA (Table 2, 1.9-fold). Paradox-

ically, whereas antisense PKC β RNA expression lowered c-jun levels in stem cells, it actually increased expression in differentiating cells (Table 2, 25.8-fold; an additional 5-fold over the effect of RA + cAMP).

The most plausible explanation for this result is that decreasing PKC β (and increasing PKC α) enhances the differentiation of F9 cells in response to RA + cAMP. This conclusion was supported by measuring type IV collagen mRNA as a marker of parietal endoderm differentiation. Previously, we demonstrated that expressing PKC α in F9 stem cells resulted in phorbol ester-induced collagen IV expression in the absence of RA-induced differentiation (11). A similar effect of PKC α expression was seen in these experiments (Fig. 2, Lane 4; Table 2, 1.1-fold over basal expression of RA + cAMP-treated F9 cells), and antisense PKC α expression prevented RA + cAMP induction of collagen IV expression (Fig. 2, Lane 10). Further treatment of phorbol ester in differentiated cells with RA + cAMP down-regulated collagen expression in general. This experiment also established a striking antagonistic effect of PKC β on collagen IV expression. Collagen IV mRNA was induced only slightly by RA + cAMP in cells constitutively expressing PKC β (Table 2, 0.3-fold), whereas antisense PKC β enhanced (Table 2, 2.4-fold) the response to RA + cAMP. In this respect, decreasing PKC β levels (Table 2, 2.4-fold) or increasing PKC α (Table 2, 3.4-fold) had the similar effects on collagen expression.

PKC α Enhanced RA and cAMP-induced Differentiation of F9 Cells into Parietal Endoderm. To determine whether the antagonistic interaction between PKC β and PKC α was limited to the collagen IV promoter or was an important component of parietal endoderm differentiation, we measured the expression of other differentiation markers in these cell lines. We used metabolic labeling and SDS-PAGE to examine the expression of basement membrane proteins, laminin A, B1/B2, and type IV collagen (Fig. 3) in the cell lines expressing various levels of PKC α and PKC β . In addition, we measured the induction of the visceral endoderm marker, TTR (20), by RNase protection (Fig. 4).

A. 5 day treatment

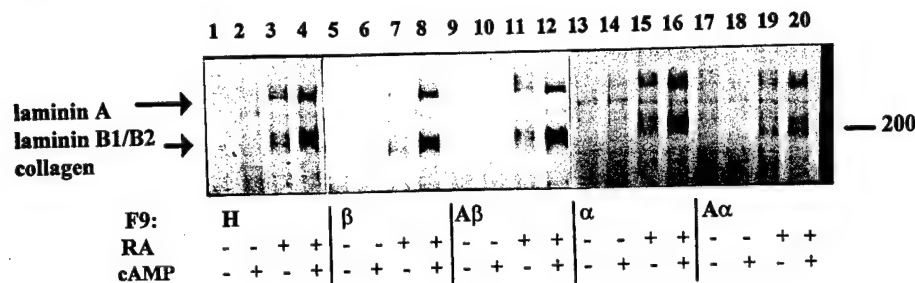


Fig. 3. PKC α or antisense PKC β expression enhances extracellular basement membrane protein synthesis in differentiated F9 cells. Basement membrane synthesis in hygromycin-resistant control (H), PKC β (β), antisense PKC β (A β), PKC α (α), and antisense PKC α (A α) transfected F9 cells was analyzed by resolving conditioned media (2.2×10^6 trichloroacetic acid insoluble cpm/lane) from radiolabeled cells on SDS-PAGE (5%). F9 cells were treated with either ethanol solvent (-RA -cAMP), 5×10^{-4} M all-trans RA (+RA), or both (+RA +cAMP) for 5 days (A) or 3 days (B). Cells were labeled with [35 S]methionine (50 μ Ci/ml) for the final 16–20 h. Right, relative migration of molecular weight standards (in thousands). Parietal endoderm differentiation is characterized by the expression of laminin A (M_r ~400,000), laminins B1 and B2, and type IV collagen (which all run at about M_r 200,000).

B. 3 day treatment

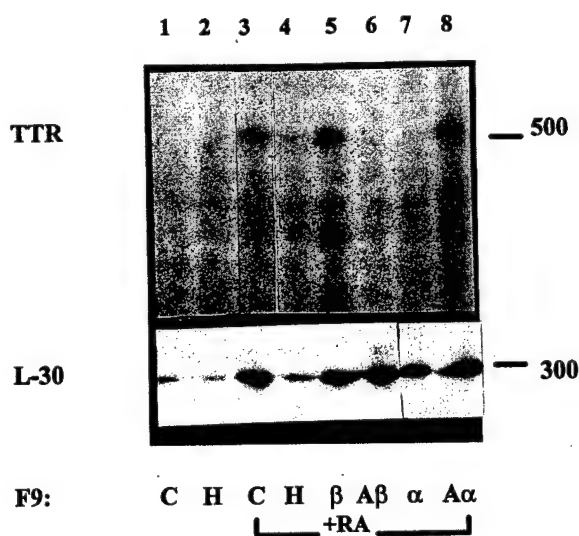
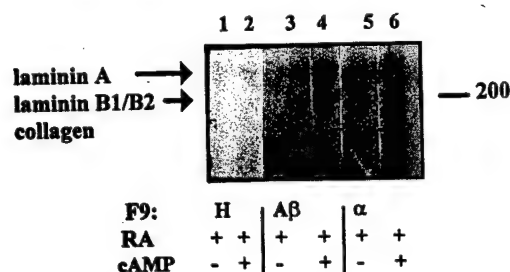


Fig. 4. PKC α and antisense PKC β expression inhibit expression of TTR, a visceral endoderm marker. F9 cells were grown as aggregates in suspension for 8 days. Total RNA was isolated, and the expression of the visceral endoderm markers TTR, retinol binding protein (data not shown; results same as TTR), and L-30 (as internal control) was measured in RNase protection assays. Lane 1, parental F9 control cells (C); Lane 2, hygromycin-resistant control cells (H); Lanes 3–8, parental F9 control (C), hygromycin-resistant control (H), PKC β (β), antisense PKC β (A β), PKC α (α), and antisense PKC α (A α) transfected cells treated with 2×10^{-8} M all-trans RA (+RA). Right, relative migration of molecular weight standards (nucleotides).

The overall pattern of basement membrane protein synthesis (Fig. 3) paralleled that reported above for the type IV collagen mRNA (Fig. 2). After 5 days of treatment with either

RA or RA and dbcAMP, in cells constitutively expressing PKC α , laminin and collagen expression were elevated relative to control cells (Fig. 3A, Lanes 3 and 4 compared with Lanes 15 and 16). This was more apparent at 3 days (Fig. 3B, compare Lanes 2 and 6). Similar results were seen in cells in which PKC β expression was reduced by antisense RNA expression (Fig. 3A, Lanes 11 and 12; Fig. 3B, Lanes 3 and 4) but not as great as increasing PKC α expression. Although overexpression of PKC β or reduced PKC α did not prevent basement membrane protein synthesis, in both cell lines the response to RA and cAMP was reduced relative to the control cells. Therefore, PKC α expression is an integral component for parietal endoderm differentiation in general and not only for the expression of type IV collagen.

F9 cells are bipotential. When aggregated and grown in the presence of RA, F9 cells acquire a visceral endoderm phenotype. The change in PKC expression seen during parietal endoderm formation does not occur during visceral endoderm formation (3, 11). By measuring TTR mRNA levels, we determined the ability of RA-treated aggregates of F9 cells transfected with PKC β , antisense PKC β , PKC α , or antisense PKC α to acquire a visceral endoderm phenotype (20). Increasing PKC α or decreasing PKC β levels inhibited the RA-induced synthesis of TTR (Fig. 4, Lanes 6 and 7). In contrast, increased expression of PKC β enhanced the expression of TTR in RA-treated F9 aggregates (Fig. 4, Lane 5).

PKC β Induced the Gradual Retrodifferentiation of the Parietal Endoderm Cell Line F9RA5. The above results strongly support a role of PKC α signaling in F9 parietal endoderm differentiation that is distinct from, and most likely antagonistic to, the role of PKC β signaling. To provide more direct evidence for a negative role of PKC β in parietal

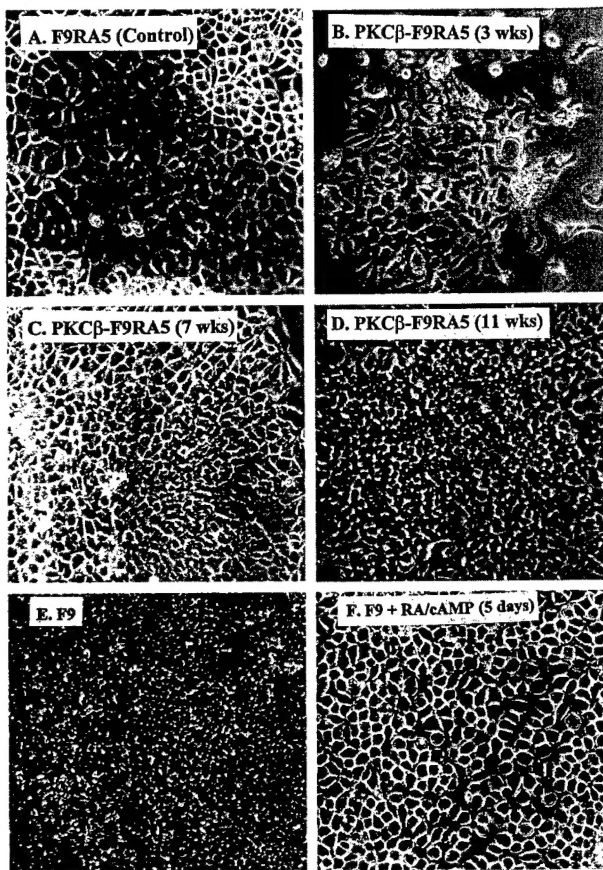


Fig. 5. PKC β induces parietal endoderm cells to redifferentiate. Exponentially growing F9RA5 control (A), PKC β -transfected F9RA5 in 3rd passage (B), 7th passage (C), and 11th passage (D), F9 control (E), and F9 cells treated with RA + cAMP for 5 days (F) were photographed using an inverted microscope ($\times 40$).

endoderm differentiation, we transfected the parietal endoderm cell line, F9RA5, with the PKC β expression plasmid. With the exception of their ability to proliferate indefinitely, the F9RA5 cells retain all morphological and biochemical characteristics of RA + cAMP-treated F9 cells, including the appropriate PKC expression pattern (Ref. 11; morphological similarity in Fig. 5, A and F). F9RA5 cells were co-transfected with plasmids encoding hygromycin (hph) resistance and either expression plasmids encoding PKC β and PKC α . Pools of hygromycin B-resistant colonies were expanded and frozen. After thawing, transfected F9RA5 cells were grown in media containing 50 μ g/ml hygromycin. F9RA5 cells expressing only the hph gene or hph gene + PKC α together retained the morphology of parental F9RA5 cells (data not shown) at all passages. However, F9RA5 cells expressing PKC β underwent a morphological transition. These cells maintained their parietal endoderm phenotype for passages 1–4 (Fig. 5B). Between passages 5 and 10, these cells underwent a morphological transition, such that around the 11th passage, the entire culture had reacquired a stem cell phenotype (Fig. 5, C and D; morphological similarity in Fig. 5, D and E). By all measurable criteria, the transition was complete. After the 11th passage, PKC β -expressing

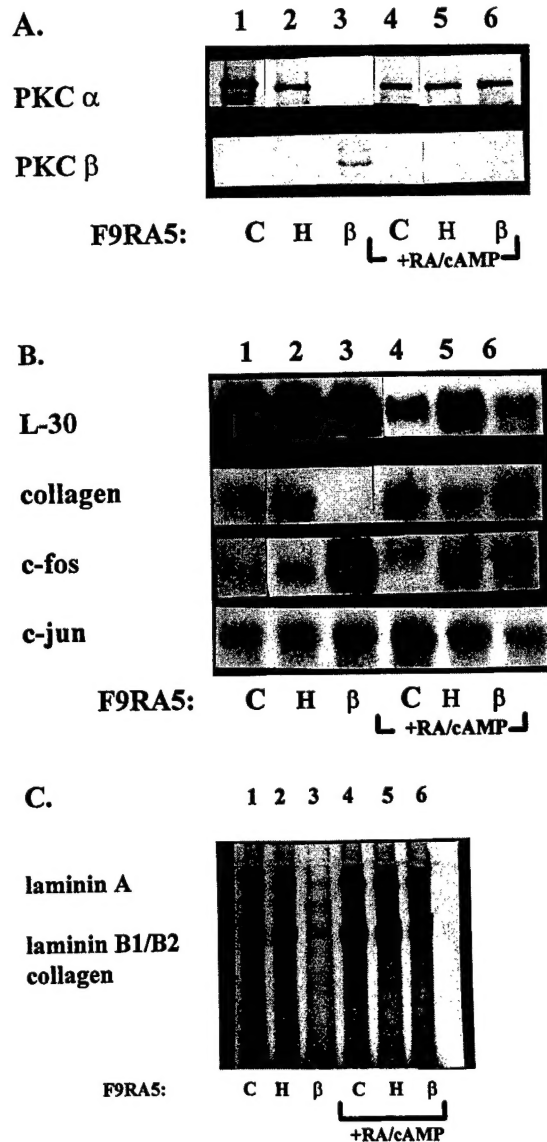


Fig. 6. Parietal endoderm marker gene expression is lost in PKC β -expressing F9RA5 cells. A, PKC α and PKC β expression in PKC β -transfected F9RA5 cells. Whole-cell protein extract (30 μ g/lane) from control (C), hygromycin-resistant control (H), and PKC β -transfected F9RA5 cells (β ; in 11th passage) was analyzed for PKC α and PKC β expression using immunoblotting as described in "Materials and Methods." In Lanes 4–6, cells were treated with 2×10^{-7} M all-trans RA and 5×10^{-4} M dbcAMP (+RA/cAMP) for 7 days. B, total RNA (20 μ g/lane) from control (C), hygromycin-resistant control (H), and PKC β -transfected F9RA5 cells (β ; in 11th passage) was analyzed for L-30, collagen IV, c-fos, and c-jun expression using Northern blotting. In Lanes 4–6, cells were treated with 2×10^{-7} M all-trans RA and 5×10^{-4} M dbcAMP (+RA/cAMP) for 7 days. L-30 is mRNA expression control. C, basement membrane synthesis in control (C), hygromycin-resistant control (H), and PKC β -transfected F9RA5 cells (β ; in 11th passage) was analyzed for protein expression of laminin A, B1, B2, and type IV collagen as described in "Materials and Methods." In Lanes 4–6, cells were treated with 2×10^{-7} M all-trans RA and 5×10^{-4} M dbcAMP (+RA/cAMP) for 7 days.

F9RA5 cells no longer expressed PKC α (Fig. 6A, Lane 3), type IV collagen (Fig. 6B, Lane 3), or laminins A/B1/B2 (Fig. 6C, Lane 3). In contrast, PKC β and c-fos were highly expressed. When the phenotypically reverted cells (PKC β ex-

pressing F9RA5 after 11th passage) were treated with RA and cAMP, PKC β expression was suppressed, and the expression of PKC α , type IV collagen, and the parietal endoderm phenotype was regained (Fig. 6, Lanes 6). Although PKC α expression was lost in PKC β -expressing F9RA5 cells, PKC α expression was promptly induced by RA and cAMP treatment (Fig. 6A, Lane 6). c-jun was constantly expressed in F9RA5 cells and was slightly elevated after RA + cAMP treatment, suggesting that PKC β expression in F9RA5 cells does not affect c-jun expression (Fig. 6B).

Discussion

In this study, we demonstrated that PKC α expression enhanced RA- and cAMP-induced differentiation of F9 mouse embryonal carcinoma cells into parietal endoderm. F9 cells that constitutively express PKC α showed an accelerated response to RA and cAMP in terms of synthesis and secretion of the basement membrane components, laminin A, B1/B2, and type IV collagen. These cells also failed to express TTR after aggregation in the presence of RA. Blocking the RA-induced expression of PKC α with antisense PKC α RNA substantially reduced induction of the parietal endoderm but not the visceral endoderm phenotype. Therefore, PKC α is critical for F9 cell differentiation and for determining which developmental pathway is used by these cells.

PKC represents a family of 11 distinct genes that show tissue and developmental stage-specific expression (16). The complexity of the PKC gene family and the specific expression patterns of members of this family led to the prediction that different PKC isozymes serve distinct, non-redundant functions. In general, PKC regulates growth and differentiation of a diverse group of cell types. In a number of these cases, induction of differentiation correlates with the differential activation of a specific PKC isozyme. Induction of PKC α expression by RA is not unique to F9 cells but also has been reported in vascular smooth muscle cells (21), B16 melanoma cells (22), NT-2 human teratocarcinoma cells (23), and human breast cancer cells (24). PKC α induction by RA (11, 21–24) appears to be a central component of differentiation in multiple cell types.

The mechanism(s) by which PKC α participates in F9 cell differentiation remains to be determined. The increase in steady-state PKC α should increase the basal level of kinase activity leading to increased phosphorylation of cellular substrates that induce the expression of parietal endoderm differentiation markers. Maciaszek *et al.* (25) and Tahayato *et al.* (26) demonstrated that phorbol esters and retinoids synergistically activate transcription of multiple promoters, possibly as a result of RA receptor phosphorylation by PKC. Phosphorylation of RA receptors increases their DNA binding activity and transcriptional activation (27, 28). The alteration of the equilibrium between phosphorylation and dephosphorylation of steroid/thyroid hormone receptor family members is proposed to be a major regulator of the balance between cell growth and differentiation (27, 29).

An alternative mechanism for the PKC α induction of F9 cell differentiation involves transcriptional regulation of parietal endoderm marker genes through phorbol ester response elements. Phorbol ester, by activating either conventional or

novel PKCs, increases the expression of genes containing TPA-responsive DNA elements in their promoter region (30). TPA-responsive DNA elements are bound by AP-1 transcription factors consisting of either homodimers of Jun family members or heterodimers of Jun and Fos family members (30). The RA- and cAMP-induced differentiation of F9 cells is accompanied by a large increase in AP-1 activity (9) and an accumulation of c-jun mRNA (Fig. 2; Ref. 11). Phorbol esters and AP-1 activity are implicated in the activation of c-jun and *laminin B2* gene transcription (9, 31). The transcriptional activation of parietal endoderm marker genes such as the laminins and type IV collagen by RA is indirect, requiring prior new protein synthesis (1, 2, 4, 32). The identity of the new RA-induced gene products that subsequently mediate parietal endoderm gene expression remains unclear. Based on the data presented here, PKC α is a strong candidate for one of these RA target genes.

The second major observation in this study is that PKC β expression is incompatible with maintenance of the parietal endoderm state. This is supported by three results: (a) PKC β expression is specifically repressed during parietal endoderm but not during visceral endoderm differentiation (11); (b) reduction of PKC β with antisense RNA accelerates the response of F9 cells to RA and cAMP (Figs. 2 and 3); and (c) reexpression of PKC β in the parietal endoderm cell line F9RA5 results in these cells reacquiring a stem cell phenotype (Figs. 5 and 6).

How PKC β accomplishes this is not clear. Several studies have linked PKC β positively with cell proliferation (19, 33). PKC β is expressed in both F9 stem cells and visceral endoderm cells that continue to proliferate but not in parietal endoderm cells that exit the cell cycle. F9RA5 cells, although they morphologically and biochemically resemble parietal endoderm cells, have retained the ability to proliferate. F9RA5 cells do not show the same degree of uncontrolled proliferation as the F9 stem cell, and appear to have distinctly different regulation of cell cycle progression (including altered cyclin D expression and Rb phosphorylation).⁴ Overexpression of PKC β in rat fibroblasts decreases their sensitivity to growth-inhibitory signals and increases their sensitivity to oncogenes (19). We have shown that PKC β is required for phorbol ester induction of c-fos in F9 cells, and that increasing PKC β levels increases c-fos expression, even in the absence of phorbol ester induction. This was especially apparent in the retrodifferentiated F9RA5 cells expressing PKC β . These data support the hypothesis that PKC β , possibly by deregulating c-fos expression, is critical in maintaining the stem cell phenotype including the characteristic uncontrolled proliferation.

Materials and Methods

Plasmid. The antisense PKC β plasmid (p β -actin SP72-As-PKC β) was constructed by ligating the 2.3-kb *Eco*RI fragment of PKC β cDNA from pJ6-PKC β (19) into the unique *Eco*RI sites of the p β -actin SP72 vector. The antisense orientation was confirmed by *Sma*I restriction digestion. The

⁴ Y. Cho *et al.*, unpublished observations.

sense and antisense PKC α plasmids (p β -actin SP72-PKC α and p β -actin SP72-As-PKC α) were constructed by ligating the 2.4-kb *EcoRI* fragment of PKC α cDNA from pMV7-PKC α into the unique *EcoRI* site of the p β -actin SP72 vector. The orientation was confirmed by *Bgl*II restriction digestion.

Cell Culture. The F9 embryonal carcinoma cells (ATCC CRL 1720) were grown on gelatin-coated tissue culture dishes in DMEM supplemented with 10% fetal bovine serum (Upstate Biotechnology, Inc., Saranac Lake, NY). Differentiation was induced by treating monolayers with 2×10^{-7} M RA and 5×10^{-4} M dbcAMP (to induce parietal endoderm) or treating aggregates plated on bacteriological Petri dishes with 2×10^{-8} M RA (to induce visceral endoderm formation). The cell line displaying stable parietal endoderm morphology and biochemical properties, F9RA5, was isolated as described previously (11).

Stable Transfection. F9 embryonal carcinoma cells were grown in DMEM containing 10% fetal bovine serum, and subconfluent cultures were cotransfected with a hygromycin resistance plasmid (p β hygro having hph cDNA) and expression plasmids encoding either PKC β (19), antisense PKC β (As PKC β), PKC α , or antisense PKC α (As PKC α) by DNA-calcium phosphate coprecipitation (34). Following transfection, cells were subjected to hygromycin B selection (150 μ g/ml) over 4 weeks. Resistant cells were maintained in DMEM, 10% fetal bovine serum, and 50 μ g/ml hygromycin B.

Western Blot Analysis for PKC Isozyme Expression. Whole-cell protein extracts were prepared with lysis buffer [20 mM Tris (pH 8), 150 mM NaCl, 10 mM sodium phosphate, 100 mM sodium vanadate, 100 mM ammonium molybdate, 10% glycerol, 1% NP40, and 0.1% SDS] as described previously (24). The protein extracts (30 μ g/lane) were separated by SDS-PAGE (10%). Following electrophoretic transfer to Hybond-C extra nitrocellulose, membranes were blocked with 5% nonfat dry milk in PBS. PKC was detected by incubating the membrane with anti-PKC isozyme antibodies (0.5 μ g/ml; affinity-purified polyclonal PKC α and PKC β antibodies; Life Technologies, Inc.), followed by extensive washing with PBST (0.05% Tween 20 in PBS) and subsequent incubation with peroxidase-conjugated secondary antibody (1:7500 dilution). PKC bands were visualized by enhanced chemiluminescence (Amersham Corp.).

RNA Isolation and Northern Blotting. The levels of specific RNAs were determined using Northern blots of total RNA isolated as described previously (24). In every case, filters were hybridized to 32 P-labeled RNA probes. Probes for L-30, c-fos, c-jun, collagen, and TTR have been described (11, 35). PKC α and PKC β RNAs were detected with riboprobes generated from human cDNA cloned into pSP72 vector. After washing in $2\times$ SSC/1% SDS and $0.2\times$ SSC/1% SDS, the Northern blots were exposed to X-ray film at -80°C with intensifying screens, and the band intensity was quantified by densitometry.

Analysis of Basement Membrane Protein Synthesis. Cells were labeled overnight with [35 S]methionine (50 μ Ci/ml; DuPont NEN). Synthesis of type IV collagen and laminin A, B1, and B2 were determined by separating 35 S-labeled extracellular proteins on 5% acrylamide gels (SDS-PAGE). Gels

were fixed, dried, and exposed to X-ray film at room temperature for 1 day.

RNAse Protection Assay. TTR RNA levels were determined using an RNAse protection assay (36). A 32 P-labeled TTR RNA probe was synthesized from TTR cDNA clone. Accurately measured amounts (10 μ g/lane) of total RNA were hybridized to the cRNA probe for 16 h at 45°C . Non-hybridized RNA and probe were removed by digestion with RNases A and T1. The hybridized RNA was subjected to separation on a denaturing 10% acrylamide gel. After fixing and drying, the gel was exposed to X-ray film with intensifying screen at -80°C .

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